Heterogenous photocatalytic inactivation of *B. stearothermophilus* endospores in aqueous suspensions under artificial and solar irradiation

Chrysanthi Berberidou[a,b], Ioannis Paspaltsis[b], Eleni Pavlidou[c], Theodoros Sklaviadis[b,d], Ioannis Poulios[a,*]

[a] Laboratory of Physical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
[b] Prion Disease Research Group, Laboratory of Pharmacology, School of Pharmacy, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
[c] Solid State Section, Department of Physics, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece
[d] Centre for Research and Technology-Hellas, Institute of Agrobiotechnology, Thessaloniki, 57001, Greece

ABSTRACT

TiO_2 mediated photocatalytic disinfection constitutes an attractive emerging technology against water-borne diseases transmitted through pathogenic microorganisms. This study demonstrates the potential of TiO_2 suspensions to fully inactivate highly resistant microorganisms, *Bacillus stearothermophilus* endospores in water, in the presence of artificial and solar irradiation. Photo-inactivation, however, in the absence of TiO_2 led to twelve times lower reaction rates and in the inactivation of only 50% of the initial endospore population, in the same time intervals. The addition of limited amounts of ferric species, as well as catalyst surface modification by Ag and Pt deposition, clearly results in enhanced reaction rates. Furthermore, electron micrographs correlate endospore inactivation and inability to reactivate in the dark with extended morphological lesions of the spore structure, caused by the photocatalytically generated reactive oxygen species.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Exposure of human health to biological contamination consists one of the most pervasive problems throughout the world. Inadequate access to clean water is directly correlated with severe problems, expected to grow worse in the coming decades: 1.2 billion people lack access to safe drinking water and millions of people die annually from diseases transmitted due to unsafe water or lack of sanitation [1,2]. In addition, the downgrading of water suitable for irrigation purposes is correlated with the existence of both current and emerging phytopathogens and the subsequent overuse of organic disinfectants [1].

Although conventional disinfection methods can eliminate an important number of pathogens, they are usually energetically demanding or require the use of harsh chemical treatments, which endanger the environment and human health. Chlorination remains one of the most popular methods of disinfecting water or wastewater, due to its' relatively high efficiency and low cost. However, the toxic, mutagenic or carcinogenic by-products produced, including trihalomethanes, halogenic acetic acids, etc. [3], as well

as its difficulty to inactivate certain pathogens commonly found in water treatment facilities, i.e. *Cryptosporidium parvum* [4], *Giardia lambia* and *Acanthamoeba* [5], has rendered chlorination as a transient solution of disinfecting water and wastewater. Negative criticism has also been raised against the use of UV-C irradiation, since it can be employed only in low turbidity water or wastewater, has high energy demands, is deprived of any residual disinfecting action and appears inadequate regarding its' potential to fully inactivate protozoan cysts [6,7].

The need to address these problems has lead to extensive research aiming to the development of novel, environmentally friendly and financially viable methods of disinfecting and purifying water and wastewater. In this context, heterogeneous photocatalytic oxidation has shown great promise in the inactivation of both current and emerging pathogens [8,9]. Since 1985 [10], bacteria [11], fungi [12], viruses [13], even prions [14] have been successfully inactivated in the presence of photocatalytically active semiconducting oxides. A key-advantage of this method is the fact that it takes place in mild environmental conditions and that it can be powered by sunlight. Several studies have also focused on fixed-TiO_2 photocatalytic disinfection, to address problems involving catalyst–solution separation [15,16].

Among the various semiconducting materials (oxides, sulfides, etc.) great attention has been drawn to TiO_2 (anatase) because of its

---

high photocatalytic activity, resistance to photocorrosion, biological inertness and low cost. General description of heterogeneous photocatalysis in the presence of TiO_2 under artificial or solar irradiation is presented in several excellent review articles [17,18]. In brief, by the irradiation of an aqueous TiO_2 suspension with light energy greater than the band gap energy of the semiconductor ($E_g > 3.2$ eV), conduction band electrons (e^-) and valence band holes (h^+) are generated. Part of the photogenerated carriers recombine in the bulk of the semiconductor, while the rest reach the surface, where the holes, as well as the electrons, act as powerful oxidants and reductants respectively. The photogenerated electrons react with the adsorbed molecular O_2 on the Ti(III)-sites, reducing it to superoxide radical anion $O_2^{\bullet-}$, while the photogenerated holes can oxidize either the organic molecules directly, or the OH^- ions and the H_2O molecules adsorbed at the TiO_2 surface to $OH^\bullet$ radicals. These radicals together with other highly oxidant species (e.g. peroxide radicals) are reported to be responsible for the primary oxidizing step in photocatalysis. The $OH^\bullet$ radicals formed on the illuminated semiconductor surface, with a standard reduction potential of 2.8 V, can easily attack the adsorbed organic molecules or those located close to the surface of the catalyst, leading finally to their complete mineralization.

The present paper demonstrates for the first time the potential of TiO_2 photocatalytic oxidation to inactivate bacterial endospores of *Bacillus stearothermophilus* under artificial and solar light, while it also aims to the enhancement of the photocatalytic efficiency by modification of TiO_2 nanoparticles. Bacterial endospores along with certain protozoans are known to be the microbial forms which demonstrate the highest resistance to harsh environmental conditions, enabling them to survive in dormant state for even thousands of years. In this dormant state, endospores undergo no detectable metabolism and exhibit a higher degree of resistance to inactivation by various physical insults, including wet and dry heat, UV and gamma radiation, extreme desiccation, vacuum, mechanical disruption and oxidizing agents [19]. This resistance is attributed, among others, to the formation of a thick multi-layered coat and to the dehydration of the protoplasm both of which protect bacterial DNA from a wide range of environmental hazards [20]. Despite their metabolic inactivity, however, spores are still capable of continually monitoring the nutritional status of their surroundings, and they respond rapidly to the presence of appropriate nutrients by germinating and resuming vegetative growth. Bacterial spores can be found in environmental samples obtained from all parts of the Earth, both above and below the surface and represent a highly successful strategy for the survival and widespread dispersal of microbial life [19]. The photocatalytic inactivation of *Clostridium perfringens* [21–23], *Bacillus subtilis* [24] and *Bacillus cereus* [25] spores in water has been reported.

Furthermore, bacterial endospores can serve as an excellent reference tool in inactivation studies, in general [26]. As far as photocatalytic oxidation is concerned, the resistance of the model microorganism increases according to the following pattern: virus < Gram-positive bacterium < Gram-negative bacterium < endospore [27]. *Escherichia coli* continues to be the most studied microorganism, since it serves as a reference organism in biology. However, the use of various strains often leads to differentiated conclusions, as far as its photocatalytic inactivation is concerned. It is well known that parameters such as type of nutrient medium, incubation temperature, phase of bacterial life cycle in which the bacterial are collected and type of aqueous media in which photocatalytic treatment is carried out may significantly influence the outcome of the study [28,29]. Thus, the comparison between different inactivation studies is often impossible. In contrast, all the above problems are trivial in the case of endospores. The initial number of endospores in suspension

remains stable, since they are in a state of metabolic hibernation, thus unable to reproduce. Furthermore, contrary to vegetative cells, they lack of most repair mechanisms. Thus, reactivation, a phenomenon commonly observed in bacterial cells [30] is significantly limited. The stability of an endospore suspension can be almost compared to that of a solution of a chemical compound and thus, to be employed as a model for the investigation of the efficiency of an inactivation process in general [26]. It must also be stressed that non-pathogenic endospores serve as excellent surrogates of highly infectious spore-forming bacterial including *Bacillus antracis* [31,32].

In this context, endospores of the species of *B. stearothermophilus* will serve as excellent indicators of the photocatalytic efficiency, since *B. stearothermophilus* in particular, is a thermophile endospore and appears as one of the most resistant types, thus, employed as an indicator of proper autoclave performance [33,34]. *B. stearothermophilus* has been isolated from hot spring waters, soil, deep sea sediments [35], while adhesion of thermophile endospores including *B. stearothermophilus* to surfaces and subsequent biofilm development is common in medical and marine environments, fresh water and water distribution units [35]. According to our knowledge, *B. stearothermophilus* has not been employed in previous photocatalytic inactivation studies and this investigation will provide new data in this field. Furthermore, by employing scanning electron microscopy (SEM), we investigate the possibility to detect any potential detrimental effect of the cell structure of this particular *Bacillus* species, induced by the oxidative attack of the photocatalytically produced reactive oxidative species (ROS).

2. Materials and methods

2.1. Preparation of B. stearothermophilus endospore suspension

B. stearothermophilus endospores were initially purchased impregnated on paper strips (ATCC 7953, Fluka, 10^6 cfu/strip). These endospores are highly resistant thermophiles and can grow at 60 °C and above. This strain is used as an indicator of proper autoclave performance and is specified by U.S. military specification MIL-S-36586 as GMP requirements of U.S. F.D.A. One strip was added in 1 L of sterile tryptic soy broth (TSB, casein peptone 17 g L^{-1}, soya peptone 3 g L^{-1}, K_2HPO_4 2.5 g L^{-1}, NaCl 5 g L^{-1}, glucose 2.5 g L^{-1}) and was incubated at 60 °C under constant agitation. After 16 h the produced vegetative cells reached stationary phase, as verified by spectrophotometric measurements at 580 nm. The vegetative cells were collected after centrifugation at $2600 \times g$ at 4 °C for 20 min and were then added to 1 L of sterile sporulation medium (yeast extract 4 g L^{-1}, nutrient broth 8 g L^{-1}, $MnCl_2$ 0.1 g L^{-1}), to induce sporulation [36]. After 6 days of incubation at 60 °C under constant agitation, endospores were collected by centrifugation at $2600 \times g$ at 4 °C for 20 min. The process described was repeated and the collected endospores were transferred to a 50 mL falcon tube, washed 5 times with cold deionized water and were then sonicated twice for 1 min (maximum power) to destroy any remaining vegetative cells. The endospores were then, washed 3 times with cold deionized water, suspended in 40 mL of deionized water and the purified spore suspension was stored at 4 °C. After staining with the Schaeffer–Fulton method [37] and enumeration employing a reverse phase microscope, it was found that the suspension consisted of at least 98% endospores. To determine the initial concentration of the prepared endospore suspension, serial dilutions were performed in triplicates and the samples were plated and incubated on TSB plates as described in Section 2.2. It was found that the initial concentration of the purified endospore suspension was 4×10^7 cfu mL^{-1}.

2.2. Enumeration of B. stearothermophilus endospores

To evaluate the efficiency of photocatalytic inactivation of *B. stearothermophilus* endospores samples were collected in duplicates and were spread on TSB plates (casein peptone $17\,g\,L^{-1}$, soya peptone $3\,g\,L^{-1}$, K_2HPO_4 $2.5\,g\,L^{-1}$, NaCl $5\,g\,L^{-1}$, glucose $2.5\,g\,L^{-1}$, bacteriological agar $15\,g\,L^{-1}$) under sterile conditions. In detail, 30 mL of TSB containing 1.5% bacteriological agar were added on 9 cm diameter sterile plates and were then incubated for 16 h at room temperature (RT), to enable detection of possible contamination. Contaminated plates were discarded. The collected samples were incubated for 5 min at 100 °C, in order to induce sporulation of endospores to vegetative cells (thermal activation) and were then centrifuged for 20 s at $12,290 \times g$. $20\,\mu L$ of each activated sample were added to 3 mL of melted TSB containing 0.7% (w/v) agaroze (pure plate method) and were spread under sterile conditions onto the plates. The plates were then incubated for 24 h at 55 °C and were enumerated by direct counting of *B. stearothermophilus* colonies.

2.3. Photocatalytic inactivation of B. stearothermophilus endospores in aqueous suspensions

For this purpose, sterile polystyrene 6 well plates with lid (Greiner) were employed. Each well served as a cylindrical 12 mL photocatalytic reactor. $166.7\,\mu L$ of the stock suspension of endospores (Section 2.1) were diluted in 10 mL of sterile distilled H_2O and were added to a single well of the plate. All the necessary catalysts and reagents were then added at the desired concentrations. The plate was illuminated by a system of 5 parallel blacklight lamps (length: 30 cm, TLD 8 W/08, Phillips), connected with a voltage regulator, placed 10 cm above the surface of the endospore suspension. The intensity of the incident irradiation at this distance was measured using a Photometer/Radiometer PMA 2100 (Solar Light Co., Glenside, Pennsylvania) equipped with a UV-A detector and found to be $9.1 \pm 0.2\,mW\,cm^{-2}$. Photocatalytic inactivation took place at room temperature, under constant magnetic stirring. Samples of $20\,\mu L$ were collected in duplicates at various time intervals in sterile Eppendorf tubes, were diluted up to a final volume of $200\,\mu L$ with sterile distilled H_2O and were kept to the dark. After the end of the photocatalytic process the samples were spread on TSB plates and enumerated as previously described (Section 2.2). $FeCl_3 \cdot 6H_2O$ was employed where Fe^{3+} was required. Photocatalytic inactivation experiments under natural light were performed similarly, using the same photocatalytic reactor, in July–August of 2009, between 13:00 and 17:00. All experiments were repeated three times, usually in sequential days, under practically identical experimental conditions. The intensity of the incident solar irradiation ranged between 2.8 and $3.2 \pm 0.1\,mW\,cm^{-2}$.

2.4. TiO_2 modification

Modification of TiO_2 (TiO_2 P-25, Degussa, anatase/rutile = 3.6/1, surface area $56\,m^2\,g^{-1}$, nonporous) was performed in a closed Pyrex cell of 600 mL capacity. The reaction mixture in the cell was magnetically stirred. The reaction vessel was fitted with a central 9 W lamp and had inlet and outlet ports for bubbling N_2 during the modification process. The spectral response of the irradiation source (Osram Dulux 9 W/78, UV-A) ranged between 350 and 400 nm, with a maximum at 366 nm. The reaction mixture consisted of an aqueous suspension of $4\,g\,L^{-1}$ TiO_2 P 25, in the presence of 5% (v/v) methanol, where the suitable amount of $AgNO_3$ (Merck) or H_2PtCl_6 $(H_2O)_6$ (Merck) was added, under constant intense stirring. The suspension was illuminated for 3 h. After the end of the photo-reduction of Ag^+ or Pt^{4+}, the modified catalyst was centrifuged for 10 min at $2600 \times g$ and was washed 5 times with distilled water. The modified catalyst was left to dry for 16 h
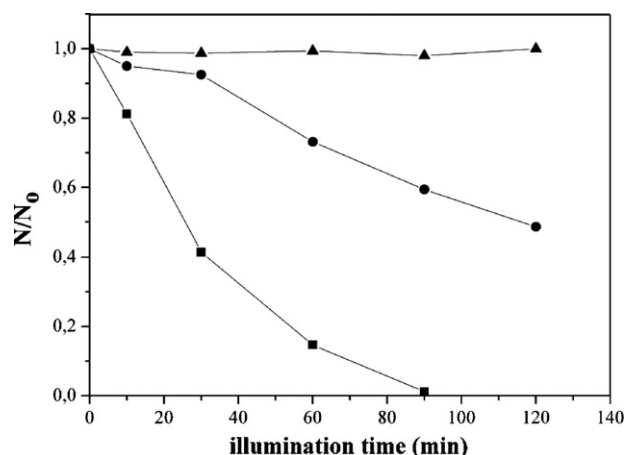
at 80 °C and was then collected in order to prepare an aqueous suspension. The suspension was sonicated, to enhance the dispersion of the modified particles and was stored at room temperature.

2.5. Scanning electron microscopy

SEM observation of *B. stearothermophilus* endospores was carried out using a JEOL, JSM-840A microscope operating at 21 kV. The samples were placed onto glass slides, dried for 16 h at room temperature and were coated with graphite twice, prior to microscopic inspection.

3. Results and discussion

Photocatalytic inactivation of *B. stearothermophilus* was conducted in aqueous endospore suspensions of $4 \times 10^5\,cfu\,mL^{-1}$, by TiO_2. Results in the presence of $0.10\,g\,L^{-1}$ TiO_2 and UV-A light are shown in Fig. 1. The amount of *B. stearothermophilus* endospores present in the suspension is plotted as a function of the irradiation time. Under these experimental conditions, inactivation of all endospores was observed within 90 min of light exposure. Inactivation in the absence of TiO_2 was also accomplished, resulting however, in an approximately 12 times lower rate constant, in comparison to the one under optimal photocatalytic conditions (Table 1). As demonstrated in Fig. 1, after 120 min of UV-A irradiation, 50% of the initial endospore population lost its ability to vegetate and form colonies. Recent studies have shown that

Fig. 1. Photocatalytic inactivation of *B. stearothermophilus* endospores in the presence of (■) $0.10\,g\,L^{-1}$ TiO_2 and UV-A light, (●) UV-A light only and (▲) $0.1\,g\,L^{-1}$ TiO_2 in the dark. $N_0 = 4 \times 10^5\,cfu\,mL^{-1}$.

Table 1
Rate constants of the photocatalytic inactivation of $4 \times 10^5\,cfu\,mL^{-1}$ endospores of *B. stearothermophilus* under various experimental conditions.

| Experimental Conditions	$k \times 10^2$ (min^{-1})	R^2
$0.10\,g\,L^{-1}$ TiO_2 P-25, UV-A (pH 7.0)	3.0 ± 0.3	0.98
$0.10\,g\,L^{-1}$ TiO_2 P-25 + $7\,mg\,L^{-1}$ Fe^{3+}, UV-A (pH 4.0)	4.4 ± 0.9	0.99
$0.10\,g\,L^{-1}$ TiO_2 P-25 + $14\,mg\,L^{-1}$ Fe^{3+}, UV-A (pH 4.0)	1.5 ± 0.4	0.98
$0.10\,g\,L^{-1}$ TiO_2 P-25 + $7\,mg\,L^{-1}$ Fe^{3+}, UV-A (pH 7.0)	3.7 ± 0.3	0.98
$0.10\,g\,L^{-1}$ TiO_2 P-25/0.1% Ag, UV-A	3.6 ± 0.6	0.95
$0.10\,g\,L^{-1}$ TiO_2 P-25/0.5% Ag, UV-A	4.0 ± 0.7	0.99
$0.10\,g\,L^{-1}$ TiO_2 P-25/1.0% Ag, UV-A	4.5 ± 0.8	0.98
$0.10\,g\,L^{-1}$ TiO_2 P-25/0.1% Pt, UV-A	5.1 ± 0.2	0.99
$0.10\,g\,L^{-1}$ TiO_2 P-25, UV-A ($I = 9.1\,mW\,cm^{-2}$)	3.0 ± 0.3	0.98
$0.10\,g\,L^{-1}$ TiO_2 P-25, solar light ($I = 2.8\,mW\,cm^{-2}$)	3.0 ± 0.6	0.93
$0.10\,g\,L^{-1}$ TiO_2 P-25, solar light ($I = 3.0\,mW\,cm^{-2}$)	3.3 ± 0.8	0.98
$0.10\,g\,L^{-1}$ TiO_2 P-25, solar light ($I = 3.2\,mW\,cm^{-2}$)	3.7 ± 0.8	0.96
UV-A ($I = 9.1\,mW\,cm^{-2}$)	0.3 ± 0.1	0.96
Solar light ($I = 2.8\,mW\,cm^{-2}$)	0.2 ± 0.1	0.95
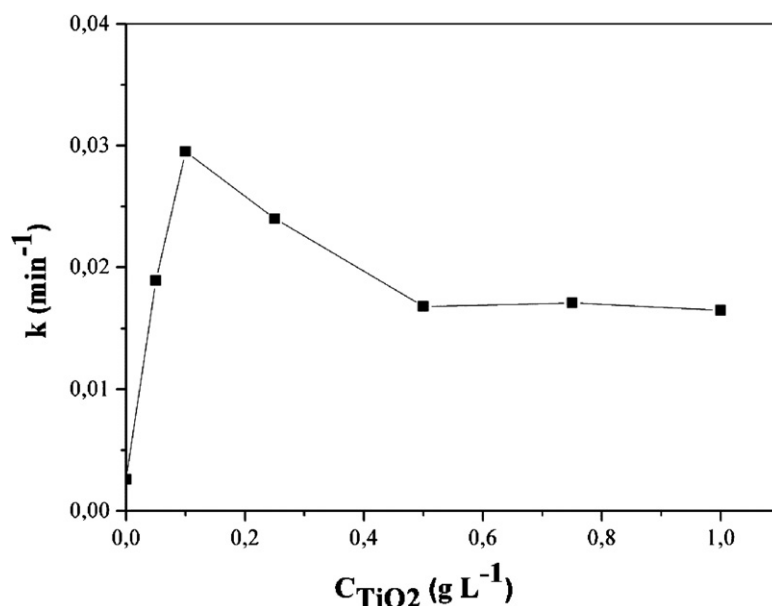
Fig. 2. Plot of the rate constant k of the photocatalytic inactivation of endospores of *B. stearothermophilus* in relation to the concentration of TiO_2. $N_0 = 4 \times 10^5$ cfu mL^{-1}.

wavelengths between 320 and 365 nm may cause DNA damage including formation of thymine and pyrimidine dimmers, along with the production of drastic oxidizing intermediates including H_2O_2 and $O_2^{\bullet-}$ [38,39], which may also cause DNA damage, mainly strand breaking or protein-DNA cross-linking, lipid superoxidation, enzyme inactivation, etc. Furthermore, although the target for spore killing by oxidizing agents is not known, there are some data implicating protein damage in these cases [19]. Indeed, most oxidizing agents appear to kill spores by causing some type of damage to spores' external layers, principally the spore's inner membrane, such that when the treated spores germinate this damaged membrane ruptures resulting in spore death. However, the precise nature of the inner membrane damage caused by oxidizing agents is not known, although it is not the oxidation of unsaturated fatty acids [40]. TiO_2 suspensions in the absence of UV-A irradiation demonstrated no sporicidal activity (Fig. 1).

The effect of varying the quantity of TiO_2 P-25 from 0.05 to 1.00 g L^{-1} on the observed initial reaction rate of *B. stearothermophilus* endospores is presented in Fig. 2. It is well known that the initial catalyst concentration is one of the key parameters affecting the initial rate of heterogenous photocatalytic oxidation, depending not only on the geometry of the photocatalytic reactor, but also on the nature of the microbial species. Here, optimal inactivation of all endospores was observed employing 0.10 g L^{-1} TiO_2, within 90 min of photocatalytic treatment, demonstrating maximum irradiation absorbance by the catalyst's nanoparticles [41]. In higher TiO_2 concentrations the rate constants were reduced, probably due to nanoparticle agglomeration [42], light scattering and reduced photon penetration phenomena [43,44].

Furthermore, we investigated the influence of the presence of Fe^{3+} during heterogenous photocatalytic inactivation of *B. stearothermophilus* endospores. It has been demonstrated that photocatalytic efficiency may be enhanced by the simultaneous presence of transition metals [45]. Here, addition of 7 mg L^{-1} Fe^{3+} (pH: 4.0) in the presence of 0.10 g L^{-1} TiO_2 and UV-A light, resulted in an increase of the reaction rate in comparison to inactivation accomplished in the absence of ferric ions (Fig. 3, Table 1). This may be attributed to the adsorption of Fe^{3+} aquacomplexes onto the surface of TiO_2 particles, which behave as electron scavengers, hindering electron–hole recombination and resulting to the reduction of ferric to ferrous ions via the photogenerated electrons.

This phenomenon may also result to an increase of the rate of electron transfer from valence to conductivity band, due to the lack of negative charge accumulation to the conductivity band. This results to an increase in the formation rate of both holes and hydroxyl radicals. Furthermore, H_2O_2 produced during the interaction of TiO_2 and UV-A reacts with ferric ions, resulting to photo-Fenton type reactions, which take place in parallel with the heterogenous photocatalytic process [30]. In addition, at acidic pH (2.5–5.0), Fe^{3+} hydrolyses giving hydroxylated species (the dominant photoreactive $Fe(OH)^{2+}$), which produce hydroxyl radicals under UV light irradiation based on the following mechanism: $Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + OH^{\bullet}$ [46,47]. Experiments in the presence of 0.10 g L^{-1} TiO_2, UV-A and pH: 4.0, in the absence of ferric species, led to similar reaction rates in comparison to the ones in neutral pH (data not shown).

Increasing the concentration of ferric ions to 14 mg L^{-1}, however, lead to a significant reduction of the reaction rate (Table 1). This may be attributed to the potential oxidation of ferric to ferrous species by either holes or hydroxyl radicals. Competition between Fe^{2+} and OH^- for interaction with the photo-generated holes may
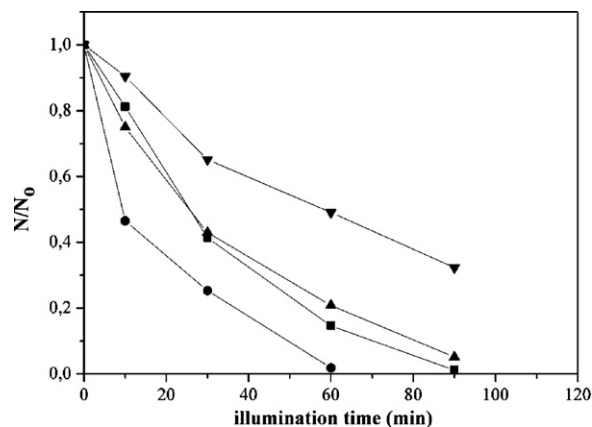

Fig. 3. Effect of the Fe^{3+} ions on the photocatalytic inactivation of *B. stearothermophilus* endospores in the presence of UV-A light: (■) 0.10 g L^{-1} TiO_2, (●) 0.10 g L^{-1} TiO_2, 7 mg L^{-1} Fe^{3+}, pH 4, (▲) 0.10 g L^{-1} TiO_2, 7 mg L^{-1} Fe^{3+}, pH 7, (▼) 0.10 g L^{-1} TiO_2, 14 mg L^{-1} Fe^{3+}, pH 4. $N_0 = 4 \times 10^5$ cfu mL^{-1}.
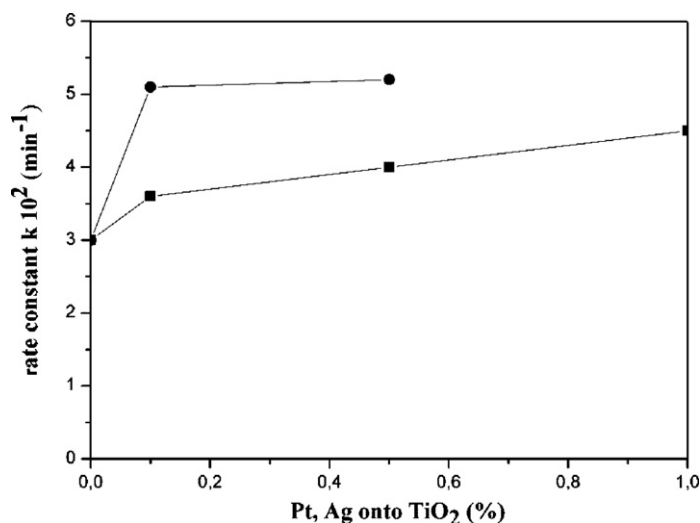
Fig. 4. Effect of TiO_2 P-25 modification with Ag or Pt deposits in the rate of heterogenous photocatalytic inactivation of endospores of *B. stearothermophilus*, in the presence of UV-A irradiation. (■) $0.10\,g\,L^{-1}$ TiO_2 P-25/Ag, (●) $0.10\,g\,L^{-1}$ TiO_2 P-25/Pt. $N_0 = 4 \times 10^5$ cfu mL^{-1}.

result to a reduction of the $OH^{\bullet}$ production rate and thus, to negative effects regarding the efficiency of the photocatalytic system [30]. Photocatalytic inactivation of *B. stearothermophilus* in the presence of $0.1\,g\,L^{-1}$ TiO_2, $7\,mg\,L^{-1}$ Fe^{3+} and UV-A, at neutral pH led to similar results in comparison to the one in the absence of ferric species, possibly due to ferric hydroxide precipitation.

To further investigate the potential enhancement of TiO_2 photocatalytic properties, we proceeded with surface modification of TiO_2 by noble metal deposition. Transition metals, such as Ag, Pt, Ni, Cu, Rh, Pd, have been studied and showed to be very effective for enhancement of TiO_2 photocatalysis [48,49]. The Fermi levels of these metals are lower than that of TiO_2; photo-excited electrons can be transferred from the conduction band to metal particles deposited on the surface of TiO_2. These metals reduce the possibility of electron–hole recombination, causing efficient charge separation and higher photocatalytic reaction rates. Modification of TiO_2 aiming to inactivate biological targets [50] has mainly focused on Ag^+ [5], which is well known for its' antimicrobial properties, while Pt and Cu have also been studied [51].

Modification of TiO_2 P-25 was conducted in the presence of Ag^+, resulting to the preparation of TiO_2 P-25/0.1% Ag, TiO_2 P-25/0.5% Ag and TiO_2 P-25/1.0% Ag. Similarly, TiO_2 P-25/0.1% Pt and TiO_2 P-25/0.5% Pt were prepared. Inactivation of *B. stearothermophilus* endospores in the presence of TiO_2 P-25/0.1% Ag, under UV-A irradiation, resulted to an increase of the photocatalytic reaction rate in comparison to the experiments performed using the unmodified catalyst (Fig. 4). Raising the amount of Ag deposited onto the TiO_2 surface further increased the respective reaction rates (Table 1). Additionally, as it can be seen in Fig. 4 and Table 1, the use of Pt modified nanoparticles, further enhanced photocatalytic inactivation, resulting to significantly higher reaction rates. However, no noteworthy differentiations were observed by increasing Pt amounts from 0.1 to 0.5%.

Photocatalytic inactivation experiments under solar irradiation were performed in July–August of 2009, always between 13:00 and 17:00 pm, employing the polystyrene plates previously described which enabled exploitation of solar irradiation above 300 nm. The light intensity under these conditions ranged between 2.8 and $3.2\,mW\,cm^{-2}$ with a mean value for all experiments of $2.9\,mW\,cm^{-2}$. Although the light intensity of solar irradiation was approximately three times lower in comparison to the artificial irradiation (UV-A) employed in the indoor experiments, the
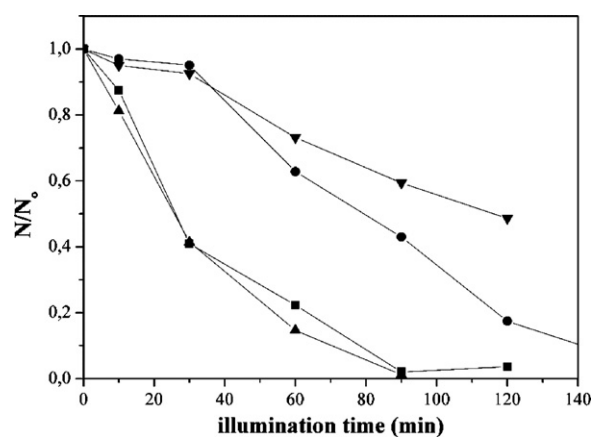

Fig. 5. Photocatalytic inactivation of the endospores *B. stearothermophilus*, in the presence of (▲) $0.10\,g\,L^{-1}$ TiO_2/UV-A ($9.1\,mW\,cm^{-2}$), (■) $0.10\,g\,L^{-1}$ TiO_2/solar light ($2.8\,mW\,cm^{-2}$), (●) solar light only ($2.8\,mW\,cm^{-2}$), (▼) UV-A only ($9.1\,mW\,cm^{-2}$). $N_0 = 4 \times 10^5$ cfu mL^{-1}.

photocatalytic reaction rates in the presence of $0.1\,g\,L^{-1}$ TiO_2 P-25 and natural light were similar or higher in comparison to the ones under artificial illumination (Table 1). It was also found, as shown in Fig. 5, that solar irradiation alone may reduce the amount of colony forming endospores. However, the reaction rate in the presence of natural light alone was remarkably lower, in comparison to the one in the simultaneous presence of TiO_2 (Table 1). The ability of solar light to cause damage to microorganisms is well known [1]. The part of solar irradiation reaching the earth's surface with the potential to cause cell damage, includes UV-B (280–320 nm) and UV-A (320–400 nm). Solar UV radiation can cause lethal and mutagenic damage to spores by direct interaction between photons and DNA. The best characterized UV damage in spore DNA is the unique thymine dimer 5-thyminyl-5,6-dihydrothymine, commonly called SP, as well as a number of less-abundant unidentified photoproducts [19]. SP has been detected in spore DNA irradiated at a number of UV wavelengths extending through the UV-C, UV-B, and UV-A portions of the spectrum, using either artificial UV sources or sunlight [38]. The presence of additional photoproducts in spore DNA exposed to solar UV has long been inferred, but the identity of some of these photoproducts has only recently begun to be elucidated [52]. Although much attention has focused on the UV-B portion of sunlight, since it is a major cause of DNA damage, recent research has clearly demonstrated that UV-A, especially wavelengths in the range from 320 to 365 nm, can also induce production of
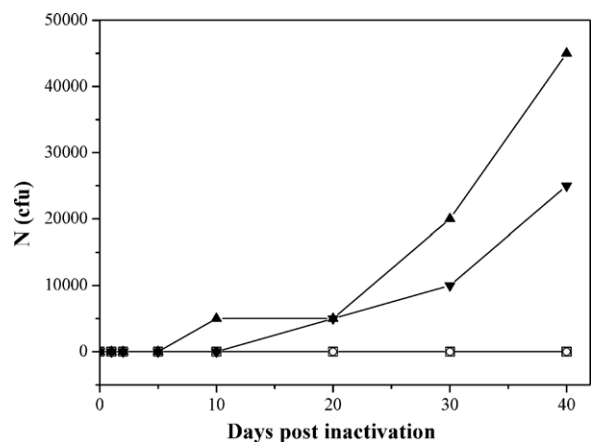

Fig. 6. Reactivation of inactivated endospores of *B. stearothermophilus* after treatment with (□) $0.10\,g\,L^{-1}$ TiO_2, UV-A, (○) $0.10\,g\,L^{-1}$ TiO_2, solar light, (▲) UV-A only, (▼) solar light only. $N_0 = 4 \times 10^5$ cfu mL^{-1}.
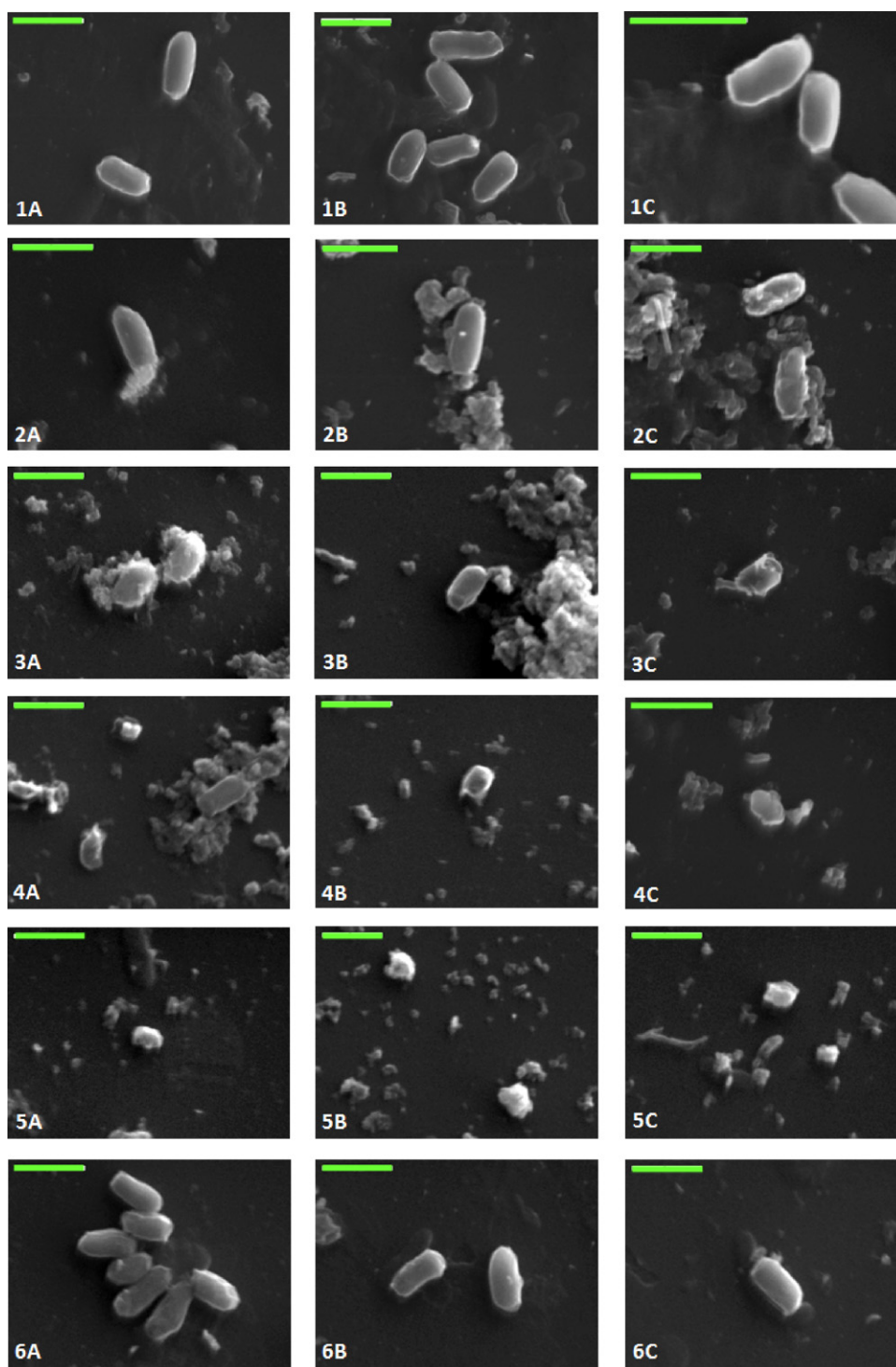
Fig. 7. SEM of *B. stearothermophilus* endospores (1): without treatment (negative controls), (2): after addition of $0.10\,g\,L^{-1}$ TiO$_2$ and stirring for 15 min in the absence of light, (3): after photocatalytic treatment in the presence of $0.10\,g\,L^{-1}$ TiO$_2$ and UV-A irradiation for 60 min, (4): after photocatalytic treatment in the presence of $0.10\,g\,L^{-1}$ TiO$_2$ and UV-A irradiation for 120 min, (5): after photocatalytic treatment in the presence of $0.10\,g\,L^{-1}$ TiO$_2$ and UV-A irradiation for 180 min, (6): after treatment in the presence of UV-A irradiation for 180 min. (A), (B), (C): different areas of the same sample or of duplicate samples. Bar: 2 μm.

pyrimidine dimers besides producing other kinds of DNA and cellular damage [38]. It has also been shown that spore DNA exposed to sunlight, especially to UV-A wavelengths, appeared to accumulate some type(s) of lethal damage which was not SP in nature [38]. Two are the potential targets by the UV-A component of sunlight in spores (i) DNA damage such as strand breaks, or modified bases. Indeed, in addition to SP, cyclobutane dimers were generated in spore DNA at physiologically relevant levels by exposure of

spores to artificial UV-B and full-spectrum sunlight but not to UV-A sunlight, whereas single-strand and double-strand breaks were induced in spore DNA exposed to full-spectrum sunlight and UV-A sunlight but not artificial UV-B [52]. From these observations it was concluded that cyclobutane pyrimidine dimers are induced in spore DNA by the UV-B portion of sunlight and that phosphodiester backbone breaks are induced in spore DNA by the UV-A wavelengths present in sunlight [52]. (ii) Damage to other, non-DNA cellular

targets. It has been well established that UV-A can generate free oxygen radicals within cells and that bacteria possess overlapping inducible stress regulons which respond to both oxidative damage and UV-A exposure, although almost nothing is yet known concerning the identity of these postulated cellular damages in spores exposed to sunlight [53].

Furthermore, any potential thermal effects of solar irradiation reported in previous studies to contribute to the inactivation of microorganisms are considered to be trivial in this study. This is due to the fact that temperatures were in all cases kept below 40 °C, suspensions were highly transparent, but mainly because *B. stearothermophilus* is a thermophile bacterium, demonstrating high resistance in temperatures up to 65 °C. Previous studies have shown that Solar Water Disinfection (SODIS), though effective against a wide range of microorganisms, failed to inactivate bacterial endospores of *B. subtilis*, resulting to only a 0.5 log reduction after 8 h of exposure to solar irradiation [54]. This fact highlights the need to promote heterogenous photocatalytic disinfection of water containing highly resistant pathogens in real applications, especially in the presence of natural light.

Several studies have reported the reactivation of bacterial cells after TiO$_2$ mediated photocatalytic oxidation, hours or even days post treatment [30]. It is not surprising that DNA repair plays a role in resistance of spores to various treatments [55]. Spores appear to contain at least some of the enzymes found in growing cells for repair of DNA damage, and DNA damage accumulated in the dormant spore will also often induce synthesis of DNA repair proteins upon subsequent spore germination [19,56]. In addition, at least one protein is uniquely present in spores, the SP lyase, which is dedicated to the repair during germination and outgrowth of the major lesion caused by UV irradiation of spores as mentioned above, the thyminyl-thymine adduct, termed the spore photoproduct (SP) [19]. Thus, in order to monitor any post inactivation regrowth effects in the present study, samples were collected and stored in the dark at room temperature for up to 40 days post inactivation. Enumeration of potentially reactivated endospores or vegetative cells was conducted as previously described. As it can be seen in Fig. 6, photocatalytic inactivation of *B. stearothermophilus* endospores under either artificial or solar light appears to have induced extended cell damage, to such an extent, that even 40 days post inactivation, no colony forming endospore can be detected. On the other hand, photo-inactivation in the presence of artificial UV-A, enabled reactivation of a part of the initial cfu, 10 days post inactivation. Similarly, solar inactivation, in the absence of TiO$_2$ resulted to reactivation of colony forming units 20 days post inactivation. This fact may be attributed to a limited oxidative attack by the photogenerated ROS, which takes place, mainly, intracellularly. Contrarily, photocatalytically induced inactivation is the result of an additional intense attack by the extracellularly generated OH$^\bullet$ and other ROS onto the exosporium and the spore coat [30], leading to its' extended damage and rupture. Possibly, photo-inactivation enabled post-inactivation repair mechanisms to be activated [19], which though limited in comparison to bacterial repair mechanisms, enabled the recovery of a small percentage of colony forming endospores.

It should also be noted that the number of endospores reactivated after UV-A induced inactivation is higher in comparison to the one caused by solar light. Thus, solar light seems to be more effective in comparison to artificial UV-A, a fact that may be attributed to the simultaneous impact of both solar UV-A and UV-B. Studies have also reported that UV-B demonstrates a more efficient sporicidal effect in comparison to UV-A [39,57].

In order to further investigate heterogeneous photocatalytic inactivation of the endospores of *B. stearothermophilus* we employed SEM. The spore suspensions were subjected to various treatments, generating interesting conclusions: (a) addition of 0.10 g L^{-1} TiO$_2$ P-25 in the spore suspension, in the absence of illumination, enabled the monitoring of adsorbed TiO$_2$ aggregates on the surface of the spore coat (Fig. 7(2A–2C)), as expected, taking into account the relative sizes of the catalyst nanoparticles and that of the spores (1.5–1.8 μm). (b) In the presence of 0.10 g L^{-1} TiO$_2$ and UV-A, the oxidative species produced, cause a detrimental effect on the shape and structure of the cells, starting from the spore coat. (c) The effect of this oxidative attack becomes more obvious as time progresses (Fig. 7(3A–4C)). After 180 min of photocatalytic treatment, detection of the known endospore structure was particularly difficult, while only fragments of the initial cells were detected, with a size in all cases smaller than 0.9 nm (Fig. 7(5A–5C)). (d) However, when inactivation of *B. stearothermophilus* endospores was accomplished by UV-A in the absence of TiO$_2$, no visible morphological lesions of the structure of the spores were observed (Fig. 7(6A–6C)), generating images similar to those of untreated endospores (Fig. 7(1A–1C)). These conclusions may correlate with the ones obtained by the reactivation study of inactivated *B. stearothermophilus* endospores. Photocatalytic inactivation resulted, undoubtedly, to extended oxidative damage of the cell structure thus, inhibiting potential cell repair mechanisms. On the other hand, photo-inactivation appears as a significantly less destructive method, which potentially enabled activation of cell repair mechanisms [58].

4. Conclusions

Our present work investigates for the first time the TiO$_2$ mediated photocatalytic inactivation of *B. stearothermophilus* endospores, a species well known for its' remarkable durability upon exposure to environmental conditions and traditional disinfection methods. Within 90 min of UV-A irradiation in the presence of 0.10 g L^{-1} TiO$_2$ suspensions, full inactivation of endospores with an initial concentration of 4×10^5 cfu mL^{-1} was accomplished. On the other hand, UV-A photo-inactivation in the absence of the catalyst, resulted to significantly lower rate constants and to the inactivation of only 50% of the initial colony forming units, within the same time duration. The enhancement of the photocatalytic efficiency was investigated by Fe^{3+} addition and surface modification by transition metals. The addition of 7 mg L^{-1} of ferric ions increased the rate constants, as well as TiO$_2$ modification by Ag and Pt, with Pt leading in all cases to higher rate constants. Furthermore, illumination of the endospore/catalyst suspension by solar irradiation led to similar or higher rate constants in comparison to the ones under artificial UV-A, while photo-inactivation with solar light alone, generated similar results to the photo-inactivation experiments under UV-A. Scanning electron microscopy enabled monitoring of severe morphological lesions on the structure of the cells caused by TiO$_2$ photocatalysis, contrarily to photo-inactivation by UV-A irradiation, which generated images similar to those of untreated cells. This observation may perhaps be correlated to the lack of dark regrowth of photocatalytically inactivated endospores, in comparison to the photo-inactivated ones. Based on our findings, we conclude that TiO$_2$ photocatalysis may serve as a highly efficient method of inactivation of recalcitrant pathogens in aqueous media, enabling natural light exploitation.

References

[1] S. Malato, P. Fernandez-Ibanez, M.I. Maldonado, J. Blanco, W. Gernjak, Catalysis Today 147 (2009) 1–59.
[2] WHO, Economic and health effects of Increasing coverage of low cost household drinking-water supply and sanitation interventions to countries off-track to meet MDG target 10, 2007.
[3] WHO, International Programme on Chemical Safety (IPCS), in: Disinfectants and disinfectant by-products, International Program on Chemical Safety (Environmental Health Criteria 216), Geneva, 1999.

[4] F. Mendez-Hermida, E. Ares-Mazas, K.G. McGuigan, M. Boyle, C. Sichel, P. Fernandez-Ibanez, Journal of Photochemistry and Photobiology B: Biology 88 (2007) 105–111.
[5] M. Sokmen, S. Degerli, A. Aslan, Experimental Parasitology 119 (2008) 44–48.
[6] D. Li, S.A. Craik, D.W. Smith, M. Belosevic, FEMS Microbiology Letters 278 (2008) 56–61.
[7] K.G. Linden, G.A. Shin, G. Faubert, W. Cairns, M.D. Sobsey, Environmental Science and Technology 36 (2002) 2519–2522.
[8] A. Markowska-Szczupak, K. Ulfig, A.W. Morawski, Catalysis Today 169 (2011) 249–257.
[9] C. McCullagh, J.M.C. Robertson, D.W. Bahnemann, P.K.J. Robertson, Research on Chemical Intermediates 33 (2007) 359–375.
[10] T. Matsunaga, R. Tomoda, T. Nakajima, H. Wake, FEMS Microbiology Letters 29 (1985) 211–214.
[11] C. Pulgarin, A.G. Rincon, Applied Catalysis B: Environmental 44 (2003) 263–284.
[12] M. Miki-Yoshida, P. Amezaga-Madrid, G.V. Nevarez-Moorillon, E. Orrantia-Borunda, FEMS Microbiology Letters 211 (2002) 183–188.
[13] H.X. Cui, J.F. Jiang, W. Gu, C.J. Sun, D.L. Wu, T. Yang, G.C. Yang, Photochemistry and Photobiology 86 (2010) 1135–1139.
[14] I. Paspaltsis, K. Kotta, R. Lagoudaki, N. Grigoriadis, I. Poulios, T. Sklaviadis, Journal of General Virology 87 (2006) 3125–3130.
[15] S.J. Khan, R.H. Reed, M.G. Rasul, BMC Microbiology 12 (2012).
[16] A. Polo, M.V. Diamanti, T. Bjarnsholt, N. Hoiby, F. Villa, M.P. Pedeferri, F. Cappitelli, Photochemistry and Photobiology 87 (2011) 1387–1394.
[17] M.N. Chong, B. Jin, C.W.K. Chow, C. Saint, Water Research 44 (2010) 2997–3027.
[18] A. Fujishima, X.T. Zhang, D.A. Tryk, Surface Science Reports 63 (2008) 515–582.
[19] W.L. Nicholson, N. Munakata, G. Horneck, H.J. Melosh, P. Setlow, Microbiology and Molecular Biology Reviews 64 (2000) 548–572.
[20] B.J. Panessawarren, G.T. Tortora, J.B. Warren, Scanning 16 (1994) 227–240.
[21] P.S.M. Dunlop, T.A. McMurray, J.W.J. Hamilton, J.A. Byrne, Journal of Photochemistry and Photobiology A: Chemistry 196 (2008) 113–119.
[22] J.R. Guimaraes, A.S. Barretto, Brazilian Journal of Chemical Engineering 20 (2003) 403–411.
[23] M. Lanao, M.P. Ormad, P. Goni, N. Miguel, R. Mosteo, J.L. Ovelleiro, Solar Energy 84 (2010) 703–709.
[24] J. Lonnen, S. Kilvington, S.C. Kehoe, F. Al-Touati, K.G. McGuigan, Water Research 39 (2005) 877–883.
[25] V. Krishna, S. Pumprueg, S.H. Lee, J. Zhao, W. Sigmund, B. Koopman, B.M. Moudgil, Process Safety and Environment Protection 83 (2005) 393–397.
[26] S. Josset, N. Keller, M.C. Lett, M.J. Ledoux, V. Keller, Chemical Society Reviews 37 (2008) 744–755.
[27] W.A. Rutala, D.J. Weber, Infection Control and Hospital Epidemiology 25 (2004) 333–341.
[28] A.G. Rincon, C. Pulgarin, Applied Catalysis B: Environmental 49 (2004) 99–112.
[29] C. Sichel, J. Blanco, S. Malato, P. Fernandez-Ibanez, Journal of Photochemistry and Photobiology A: Chemistry 189 (2007) 239–246.
[30] A.G. Rincon, C. Pulgarin, Catalysis Today 124 (2007) 204–214.
[31] G.K. Prasad, G.S. Agarwal, B. Singh, G.P. Rai, R. Vijayaraghavan, Journal of Hazardous Materials 165 (2009) 506–510.
[32] J. Zhao, V. Krishna, B. Hua, B. Moudgil, B. Koopman, Journal of Photochemistry and Photobiology B: Biology 94 (2009) 96–100.
[33] H. Albert, D.J.G. Davies, L.P. Woodson, C.J. Soper, Journal of Applied Microbiology 85 (1998) 865–874.
[34] W.A. Rutala, D.J. Weber, Infection Control and Hospital Epidemiology 17 (1996) 773.
[35] S.A. Burgess, D. Lindsay, S.H. Flint, International Journal of Food Microbiology 144 (2010) 215–225.
[36] P.J. Thompson, O.A. Thames, Applied Microbiology 15 (1967) 975–979.
[37] A.B. Schaeffer, M.D. Fulton, Science 77 (1933) 194.
[38] R.M. Tyrrell, Photochemistry and Photobiology 27 (1978) 571–579.
[39] Y. Xue, W.L. Nicholson, Applied and Environment Microbiology 62 (1996) 2221–2227.
[40] P. Setlow, Journal of Applied Microbiology 101 (2006) 514–525.
[41] S. Parra, J. Olivero, C. Pulgarin, Applied Catalysis B: Environmental 36 (2002) 75–85.
[42] S. Sakthivel, B. Neppolian, M.V. Shankar, B. Arabindoo, M. Palanichamy, V. Murugesan, Solar Energy Materials and Solar Cells 77 (2003) 65–82.
[43] N. Daneshvar, D. Salari, A.R. Khataee, Journal of Photochemistry and Photobiology A: Chemistry 157 (2003) 111–116.
[44] J.M. Herrmann, C. Guillard, Comptes Rendus De L'Académie Des Sciences Serie Ii Fascicule C-Chimie 3 (2000) 417–422.
[45] M.I. Litter, Applied Catalysis B: Environmental 23 (1999) 89–114.
[46] P. Mazellier, M. Bolte, Chemosphere 35 (1997) 2181–2192.
[47] M.H. Zhou, L.C. Lei, Chemosphere 63 (2006) 1032–1040.
[48] S. Rehman, R. Ullah, A.M. Butt, N.D. Gohar, Journal of Hazardous Materials 170 (2009) 560–569.
[49] J.C. Zhao, C.C. Chen, W.H. Ma, Topics in Catalysis 35 (2005) 269–278.
[50] A. Vohra, D.Y. Goswami, D.A. Deshpande, S.S. Block, Journal of Industrial Microbiology and Biotechnology 32 (2005) 364–370.
[51] T. Sato, M. Taya, Biochemical Engineering Journal 30 (2006) 199–204.
[52] T.A. Slieman, W.L. Nicholson, Applied and Environment Microbiology 66 (2000) 199–205.
[53] R.M. Tyrrell, in: F. Urbach (Ed.), Biological Responses to Ultraviolet-A Radiation, Valdenmar Publishing, Overland Park, Kansas, 1992, pp. 59–64.
[54] M. Boyle, C. Sichel, P. Fernandez-Ibanez, G.B. Arias-Quiroz, M. Iriarte-Puna, A. Mercado, E. Ubomba-Jaswa, K.G. McGuigan, Applied and Environment Microbiology 74 (2008) 2997–3001.
[55] P. Setlow, Annual Review of Microbiology 49 (1995) 29–54.
[56] B. Setlow, P. Setlow, Journal of Bacteriology 178 (1996) 3486–3495.
[57] N. Munakata, Journal of Radiation Research 30 (1989) 338–351.
[58] R. Moeller, E. Stackebrandt, G. Reitz, T. Berger, P. Rettberg, A.J. Doherty, G. Horneck, W.L. Nicholson, Journal of Bacteriology 189 (2007) 3306–3311.